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"Novel membrane-bound metalloprotease NEP II and the use thereof for screening inhibitors useful in therapy"

5 The subject of the present invention is a novel membrane-bound metalloprotease called NEP II and the use thereof, in particular for screening inhibitors useful in therapy.

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10 Membrane-bound metalloproteases such as neprilysin (NEP I, EC 3.4.24.11) play an important role in the activation or inactivation of neuronal or hormonal peptide messengers. Their selective inhibition by synthetic compounds has already led to medicinal products which are commonly used in therapeutics, or  
15 which are in the process of clinical development, in particular in the gastroenterological (Baumer et al., Gut, 1992, 33: 753-758) and cardiovascular (Gros et al., Proc. Natl. Acad. Sci. USA, 1991, 88: 4210-4214) fields. The isolation of the cDNAs of genes of novel  
20 related metalloproteases is likely to enable the development of novel classes of specific inhibitors with promising therapeutic uses. It is in this way that the cloning and the expression of the endothelin-converting enzyme (ECE) gene (Xu et al., Cell, 1994,  
25 78: 473-485) allowed the development of inhibitors which are potentially useful in certain cardiovascular disorders.

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30 The authors of the present invention have revealed a novel membrane-bound metalloprotease belonging to the ECE/NEP/Kell family (Lee S. et al., 1991, PNAS 88(14): 6353-57), which they have called NEP II.

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35 A subject of the present invention is thus an isolated polypeptide comprising an amino acid sequence chosen from the sequence SEQ ID No. 2 or SEQ ID No. 4, a sequence derived from or homologous to said sequence SEQ ID No. 2 or SEQ ID No. 4, and a biologically active fragment of said sequence SEQ ID No. 2 or SEQ ID No. 4,

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said isolated polypeptide being referred to as "NEP II".

The sequence SEQ ID No. 2 is the amino acid sequence of NEP II identified in rats.

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The sequence SEQ ID No. 4 is an amino acid sequence (partial) of NEP II identified in humans.

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The term "derived" polypeptide is intended to mean any polypeptide resulting from a modification of genetic and/or chemical type of the sequence SEQ ID No. 2 or SEQ ID No. 4, i.e. by mutation, deletion, addition, substitution and/or chemical modification of at least one amino acid, or any isoform having a sequence identical to the sequence SEQ ID No. 2 or SEQ ID No. 4, but containing at least one amino acid in the D form.

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Said substitutions are preferably conservative substitutions, i.e. substitutions of amino acids of the same class, such as substitutions of amino acids with uncharged side chains (such as asparagine, glutamine, serine, threonine or tyrosine), of amino acids with basic side chains (such as lysine, arginine or histidine), of amino acids with acidic side chains (such as aspartic acid or glutamic acid) or of amino acids with apolar side chains (such as glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan or cysteine).

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The term "homologous" polypeptide is intended to mean more particularly any polypeptide which can be isolated from mammalian species other than rats or humans.

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Said homologous polypeptides show preferably greater than 70%, even more preferably greater than 75%, sequence homology with the complete sequence SEQ ID No. 2 or SEQ ID No. 4, the homology being particularly high in that portion of said polypeptide containing the active site.

The homology is generally determined using a sequence analysis software package (for example, Sequence Analysis Software Package of the Genetics

5 To this end, it may be necessary to artificially  
introduce "gaps" into the sequence. Once the optimum  
alignment has been produced, the degree of homology  
(i.e. identity) is established by recording all the  
positions for which the amino acids of the two compared  
0 sequences are identical, with respect to the total  
number of positions.

Said polypeptides derived from or homologous to, or the polypeptide fragments of, the polypeptide of sequence SEQ ID No. 2 or SEQ ID No. 4 are biologically active, i.e. they have biological properties identical or similar of the biological properties of the NEP II polypeptide of sequence SEQ ID No. 2 or SEQ ID No. 4, namely metalloprotease activity.

The preferred polypeptide fragments comprise the sequence of the active site responsible for binding the zinc atom which is essential for the catalysis. This active site has been identified as encompassing the HEX<sub>1</sub>X<sub>2</sub>H, X<sub>1</sub> and X<sub>2</sub> residues representing varied amino acids. It is in particular the HEITH sequence (amino acids 608 to 612 of the sequence SEQ ID No. 2) in the NEP/II polypeptide in rats and humans.

A subject of the present invention is also an isolated nucleic acid comprising a nucleotide sequence chosen from the sequence SEQ ID No. 1 or SEQ ID No. 3, a sequence derived from or homologous to said sequence SEQ ID No. 1 or SEQ ID No. 3, and the complementary sequences thereof.

The sequence SEQ ID No. 1 is the cDNA sequence comprising the coding frame for NEP II identified in rats.

The sequence SEQ ID No. 3 is the cDNA sequence comprising (partially) the coding frame for NEP II identified in humans.

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5 The term "derived" nucleotide sequence is intended to mean any nucleotide sequence encoding a polypeptide derived from NEP II as defined above, i.e. a sequence resulting from a modification of the sequence SEQ ID No. 1 or SEQ ID No. 3, in particular by mutation, deletion, addition or substitution of at least one nucleotide. Included in particular are the sequences which are derived from the sequence SEQ ID No. 1 or SEQ ID No. 3 by degeneracy of the genetic  
10 code.

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15 The term "homologous" sequence is intended to mean more particularly any nucleotide sequence encoding an NEP II polypeptide homologous to the NEP II polypeptide of sequence SEQ ID No. 2 or SEQ ID No. 4 in mammalian species other than rats or humans.

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20 Such a homologous sequence has preferably greater than 70%, even more preferably greater than 75%, homology with the sequence SEQ ID No. 1 or SEQ ID No. 3, the homology being particularly high in the central portion of the sequence encoding the NEP II polypeptide.

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25 Preferably, such as homologous nucleotide sequence hybridizes specifically with the sequences which are complementary to the sequence SEQ ID No. 1 or No. 3, under stringent conditions. The parameters which define the stringency conditions depend on the temperature at which 50% of the paired strands separate (Tm).

30 For sequences comprising more than 30 bases, Tm is defined by the equation:  $Tm = 81.5 + 0.41(\%G+C) + 16.6 \log(\text{concentration of cations}) - 0.63(\% \text{formamide}) - (600/\text{number of bases})$  (Sambrook et al., Molecular Cloning, A laboratory manual, Cold Spring Harbor laboratory Press, 1989, pages 9.54-9.62).

35 For sequences more than 30 bases long, Tm is defined by the equation:  $Tm = 4(G+C) + 2(A+T)$ .

Under suitable stringency conditions, under which the nonspecific sequences do not hybridize, the hybridization temperature is approximately 5 to 30°C,

preferably 5 to 15°C, below  $T_m$ , even more preferably 5 to 10°C below  $T_m$  (high stringency), and the hybridization buffers used are preferably solutions with high ionic strength, such as a 6xSSC solution for example.

5 The nucleotide sequences according to the invention can be used for producing a recombinant NEP II protein according to the invention, according to techniques for producing recombinant products, known to persons skilled in the art.

10 An effective system for producing a recombinant protein must have a vector, for example of plasmid or viral origin, and a compatible host cell.

15 The cellular host can be chosen from prokaryotic systems such as bacteria, or eukaryotic systems such as, for example, yeasts, insect cells or mammalian cells, for instance CHO cells (Chinese hamster ovary cells), or any other advantageously available system.

20 The vector should comprise a promoter, translation initiation and termination signals, and the suitable transcription regulation regions. It should be able to be integrated into the cell and can optionally have specific signals determining the secretion of the translated protein.

25 These various control signals are chosen according to the cellular host used. For this purpose, the nucleotide sequences according to the invention can be inserted into vectors which replicate autonomously within the chosen host, or vectors which integrate in the chosen host. Such vectors will be prepared according to the methods commonly used by persons skilled in the art, and the clones resulting therefrom can be introduced into a suitable host by standard methods, such as for example electroporation.

35 Examples of vectors of interest are the plasmids pCDNA 3.1, PCR2.1 (Invitrogen), or pMbac (Stratagene).

The invention is aimed toward the cloning and/or expression vectors containing a nucleotide sequence according to the invention, and is also aimed toward the host cells transfected with these vectors.

5 These cells can be obtained by introducing into host cells a nucleotide sequence inserted into a vector as defined above, and then culturing said cells under conditions which allow the replication and/or expression of the transfected nucleotide sequence.

10 These cells can be used in a method for producing a recombinant polypeptide according to the invention.

15 The method for producing a polypeptide of the invention in recombinant form is itself included in the present invention, and is characterized in that the transfected cells are cultured under conditions which allow the expression of a recombinant polypeptide according to the invention, and in that said recombinant polypeptide is recovered.

20 The purification methods used are known to persons skilled in the art. The recombinant polypeptide can be purified from cell lysates and extracts, or from the culture medium supernatant, by methods used separately or in combination, such as fractionation, chromatography methods, or immunoaffinity techniques  
25 using monoclonal antibodies or polyclonal serum, etc.

A subject of the present invention is also the nucleotide probes which are capable of hybridizing strongly and specifically with a nucleic acid sequence,  
30 of a genomic DNA or of a messenger RNA, encoding a polypeptide according to the invention. The suitable hybridization conditions correspond to the temperature and ionic strength conditions conventionally used by persons skilled in the art (Sambrook et al., 1989),  
35 preferably to conditions of high stringency, i.e. temperature conditions between ( $T_m$  minus  $5^\circ\text{C}$ ) and ( $T_m$  minus  $15^\circ\text{C}$ ) and even more preferably to temperature conditions between  $T_m$  and ( $T_m$  minus  $10^\circ\text{C}$ ) (high stringency).

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1. The first part of the paper is devoted to a review of the literature on the effects of the 1997-1998 Asian financial crisis on the economies of the Asian countries. The second part of the paper is devoted to a review of the literature on the effects of the 1997-1998 Asian financial crisis on the economies of the Asian countries. The third part of the paper is devoted to a review of the literature on the effects of the 1997-1998 Asian financial crisis on the economies of the Asian countries.

culturing described by Köhler and Milstein (Nature, 1975, vol. 256, pp 495-497).

The antibodies can be chimeric antibodies, humanized antibodies or Fab and F(ab')<sub>2</sub> fragments. They can also be in the form of labeled antibodies or immunoconjugates.

The antibodies according to the invention are particularly useful for detecting the presence of NEP II.

A subject of the present invention is therefore a method for immunologically detecting NEP II in a cell or tissue sample or in cells or a tissue, comprising the steps consisting in:

- bringing said cell or tissue sample, said cells or said tissue into contact with a detectable antibody according to the invention;

- detecting the presence of said antibody, which is an indication of the presence of the NEP II polypeptide.

The term "detectable antibody" is intended to mean either an antibody labeled with a detectable group, such as a group which is radioactive, enzymatic, fluorogenic or fluorescent, or an antibody to which another antibody, which is itself labeled in a detectable manner is bound.

The antibodies according to the invention can thus make it possible to evaluate overexpression of the <sup>NEP</sup> ~~[[lacuna]]~~ II polypeptide, which may be an indication of neuroendocrine tumour cells in particular.

A subject of the invention is also a method for identifying compounds which are substrates for the NEP II polypeptide as defined above, in which said compounds, optionally labeled, are brought into contact with the NEP II polypeptide, and the cleavage of said compounds by NEP II, which is an indication of the metalloprotease activity of NEP II toward said substrate compounds, is evaluated.

Such substrates specific for NEP II can in particular be used in a method for detecting the



metalloprotease activity of NEP II in a cell or tissue sample or in cells or a tissue, comprising the steps consisting in:

5 - bringing said cell or tissue sample, said cells or said tissue into contact with a compound which is a substrate for the NEP II polypeptide, obtained according to the invention, said substrate compound being optionally labeled;

10 - evaluating the cleavage of said substrate compound, which is an indication of the metalloprotease activity of NEP II.

Cells which can be thus assayed are especially cells transfected with a polynucleotide encoding the NEP II polypeptide as defined above. Tissue extracts which can be assayed are especially testicle membranes, which are particularly rich in NEP II metalloprotease.

A subject of the invention is, moreover, a method for screening compounds which are capable of inhibiting the metalloprotease activity of the NEP II polypeptide according to the invention, in which said compounds are brought into contact with said NEP II polypeptide and the degree of inhibition of the metalloprotease activity of NEP II is evaluated.

25 The compounds capable of inhibiting the metalloprotease activity of NEP II are preferably short peptides of 2 or 3 natural or modified amino acids.

30 The synthetic peptides identified as inhibitors of the metalloprotease activity of NEP II by this screening method can be coupled to a zinc-chelating group, such as thiol, phosphate or hydroxamic acid groups, according to the conventional techniques known to persons skilled in the art. The inhibitor compound obtained is a good candidate as an active principle of a medicinal product, in combination with a  
35 pharmaceutically acceptable vehicle. Said chelating group can optionally be transiently protected, for example with a thiol ester, so as to improve the bioavailability of said active principle.

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The NEP II polypeptide according to the invention is particularly useful for screening compounds which are inhibitors of the metalloprotease activity of NEP II and which are useful for manufacturing a medicinal product intended for treating disorders involving peptide transmissions in which NEP II participates.

10 Among the disorders under consideration, mention may be made in particular of cardiovascular and neurodegenerative diseases, growth disorders of endocrine origin, disturbances of the hypothalamo-hypophysial axis and endocrine conditions. More particularly targeted are disorders affecting the metabolism of neurohormones or factors of the  
15 corticotropic sphere.

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The compounds which are substrates for NEP II or which are inhibitors of the metalloprotease activity of NEP II, obtained according to the methods described above, can also be useful for detecting the NEP II protein.  
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A subject of the present invention is therefore also a method for detecting NEP II in a cell or tissue sample or in cells or a tissue, comprising the steps consisting in:

25 - bringing said cell or tissue sample, said cells or said tissue into contact with a compound which is a substrate for the NEP II polypeptide, obtained as defined above, or with a compound which is an inhibitor of the metalloprotease activity of NEP II, obtained  
30 according to the screening method as defined above, said substrate compound or said inhibitor compound being labeled;

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- detecting the presence of said substrate compound or of said inhibitor compound, which is an indication of the presence of the NEP II polypeptide.  
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The term "labeled substrate compound" or "labeled inhibitor" is intended to mean a substrate compound or an inhibitor compound which is labeled in a detectable manner, for example, with a group which is

radioactive, enzymatic, fluorogenic or fluorescent, etc.

The following examples illustrate the invention without limiting it.

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**EXAMPLE 1:**

**Cloning the cDNA encoding NEP II in rats**

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10 Degenerate oligonucleotides were obtained based on the alignment of the peptide sequences of the ECE, NEP I and Kell enzymes, and on the delimitation of the regions of strong homology.

15 The total RNA of various rat tissues (brain, intestine and testicles) was subjected to reverse transcription (RT) and amplified by polymerase chain reaction (PCR), using a pair of degenerate oligonucleotides, over the N-terminal region rich in cysteine residues:

The sequences of these degenerate oligonucleotides are as follows:

20 DCYS2 CCC AAG (G/T)CG (A/G)G(A/G) CTG GTC  
DCYS3 T(A/T)(C/T) GC(A/C/T/G) GG(A/T) GG(A/C) TGG

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25 This made it possible to amplify a 420-base pair fragment from the testicle RNAt, encoding an open reading frame which has 76% homology with the NEP I protein. This sequence was completed by 3' and 5' RACE (rapid amplification of cDNA ends), using RNAt from brain and from testicles. The sequences were confirmed by verifying five different clones for each tissue and each amplification. The complete cDNA (SEQ ID No. 1)  
30 was then cloned into the vectors PCR2.1 and pCDNA3.1 (Invitrogen).

**EXAMPLE 2:**

**Characteristics of the rat NEP II polypeptide**

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The novel gene isolated encodes a 774-amino acid protein (SEQ ID No. 2) which, besides strong homologies with the NEP I, ECE and Kell enzymes (52%, 40% and 28% amino acid identity, respectively), has the consensus sequence of the HEXXH active site, a

transmembrane region (amino acids 24 to 40 in the  
sequence SEQ ID No. 2) followed by four cysteine  
residues which are characteristic of this family, and  
seven potential glycosylation sites. Three alternative  
5 splicings were identified by sequencing the RACE  
products and by RT-PCR. One of these alternative  
splicings eliminates a potential glycosylation site and  
might affect the transit of the protein to the surface  
of the cell, or its activity. Each splicing  
10 corresponds, moreover, to an exon of NEP I, which  
suggests a similar gene structure. These data  
demonstrate that this novel enzyme belongs to the  
family of ECE/NEP/Kell metalloproteases. Its notable  
homology with NEP I led to it being named NEP II.

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EXAMPLE 3:

Cloning the cDNA encoding NEP II in humans

In order to clone the human homologue of  
NEP II, two oligonucleotides were designed, based on  
20 the protein sequence of rat NEP II. The sequences were  
chosen, on the one hand, for their low degeneracy (such  
as, for example, a tryptophan, represented by a single  
codon in the genetic code) and, on the other hand, for  
their degree of conservation (such as the zinc binding  
25 site).

1- (H)EITHFD (SEQ ID No. 28) or 5' - CGA GAT CAC ACA TGG CTT TGA  
TGA - 3' (S) (SEQ ID No. 22)

2- QVWCGS (SEQ ID No. 29) or 5' - GGA CCC ACA CCA CAC CTG - 3' (AS)  
(SEQ ID n° 23)

A polymerase chain reaction was carried out on  
human hippocampe cDNA obtained from a library  
(Stratagene), and a 330-bp band was amplified,  
subcloned and sequenced (SEQ ID No. 3). The sequence  
obtained shows 82% sequence homology with rat NEP II,  
which makes it possible to assert that it encodes the  
human homologue.

The presence of the HEITH zinc binding site was confirmed by 5' RACE using the human-specific HNII-2 and HNII-3 oligonucleotides. Similarly, the HNII-1 and HNII-2 oligonucleotides will enable the amplification of the 3' region by the 3' RACE technique.

- HNII-1 5'- CGG CCT GGA TCT CAC CCA TGA G - 3' (SEQ IDNo.24)
- HNII-2 5'- CTG ACT GCT CC~~G~~ GGA AGT GCT GGG TG - 3' (SEQ IDNo.25)
- HNII-3 5'- GAG CAG CTC TTC TTC ATC - 3' (SEQ IDNo.26)
- HNII-4 5'- CTC CAC CAA TCC ATC ATG TTG C - 3' (SEQ IDNo.27).

EXAMPLE 4:

NEP II tissue expression

Northern blot and RT-PCR studies show that NEP II is encoded by a 2.8-Kb transcript which is very highly expressed in rat testicles, and moderately expressed in the heart, the liver, the digestive sytem and the brain. Semi quantitative RT-PCR studies show a similar expression profile in these tissues and a predominance of the long forms.

All these characteristics indicate clearly that the protein identified for the first time is a membrane-bound metalloprotease (ectoprotease) responsible for the metabolism of neuronal and/or hormonal messenger peptides.

The native NEP II polypeptide is expressed in a heterogeneous manner in the nervous system, the glands (hypophyses, testicle), the digestive apparatus (small intestine in particular) and the cardiovascular system (heart in particular).

In situ hybridization techniques also indicate a high expression of the NEP II protein in neurons and adenohypophysial cells expressing the gene for POMC (propiomelanocortin), which is the precursor of ACTH.

These locations indicate the participation of NEP II in the proteolysis of hormones and of peptide neurotransmitters, or of their precursors, coming from or acting on these diverse organs. It consequently becomes advantageous, for therapeutic purposes, to affect the corresponding peptide transmissions by inhibiting NEP II.

1. *Principles of the Law of the Sea*, 1982, 1988, 1992, 1996, 2000, 2004, 2008, 2012, 2016, 2020, 2024, 2028, 2032, 2036, 2040, 2044, 2048, 2052, 2056, 2060, 2064, 2068, 2072, 2076, 2080, 2084, 2088, 2092, 2096, 2100, 2104, 2108, 2112, 2116, 2120, 2124, 2128, 2132, 2136, 2140, 2144, 2148, 2152, 2156, 2160, 2164, 2168, 2172, 2176, 2180, 2184, 2188, 2192, 2196, 2200, 2204, 2208, 2212, 2216, 2220, 2224, 2228, 2232, 2236, 2240, 2244, 2248, 2252, 2256, 2260, 2264, 2268, 2272, 2276, 2280, 2284, 2288, 2292, 2296, 2300, 2304, 2308, 2312, 2316, 2320, 2324, 2328, 2332, 2336, 2340, 2344, 2348, 2352, 2356, 2360, 2364, 2368, 2372, 2376, 2380, 2384, 2388, 2392, 2396, 2400, 2404, 2408, 2412, 2416, 2420, 2424, 2428, 2432, 2436, 2440, 2444, 2448, 2452, 2456, 2460, 2464, 2468, 2472, 2476, 2480, 2484, 2488, 2492, 2496, 2500, 2504, 2508, 2512, 2516, 2520, 2524, 2528, 2532, 2536, 2540, 2544, 2548, 2552, 2556, 2560, 2564, 2568, 2572, 2576, 2580, 2584, 2588, 2592, 2596, 2600, 2604, 2608, 2612, 2616, 2620, 2624, 2628, 2632, 2636, 2640, 2644, 2648, 2652, 2656, 2660, 2664, 2668, 2672, 2676, 2680, 2684, 2688, 2692, 2696, 2700, 2704, 2708, 2712, 2716, 2720, 2724, 2728, 2732, 2736, 2740, 2744, 2748, 2752, 2756, 2760, 2764, 2768, 2772, 2776, 2780, 2784, 2788, 2792, 2796, 2800, 2804, 2808, 2812, 2816, 2820, 2824, 2828, 2832, 2836, 2840, 2844, 2848, 2852, 2856, 2860, 2864, 2868, 2872, 2876, 2880, 2884, 2888, 2892, 2896, 2900, 2904, 2908, 2912, 2916, 2920, 2924, 2928, 2932, 2936, 2940, 2944, 2948, 2952, 2956, 2960, 2964, 2968, 2972, 2976, 2980, 2984, 2988, 2992, 2996, 3000, 3004, 3008, 3012, 3016, 3020, 3024, 3028, 3032, 3036, 3040, 3044, 3048, 3052, 3056, 3060, 3064, 3068, 3072, 3076, 3080, 3084, 3088, 3092, 3096, 3100, 3104, 3108, 3112, 3116, 3120, 3124, 3128, 3132, 3136, 3140, 3144, 3148, 3152, 3156, 3160, 3164, 3168, 3172, 3176, 3180, 3184, 3188, 3192, 3196, 3200, 3204, 3208, 3212, 3216, 3220, 3224, 3228, 3232, 3236, 3240, 3244, 3248, 3252, 3256, 3260, 3264, 3268, 3272, 3276, 3280, 3284, 3288, 3292, 3296, 3300, 3304, 3308, 3312, 3316, 3320, 3324, 3328, 3332, 3336, 3340, 3344, 3348, 3352, 3356, 3360, 3364, 3368, 3372, 3376, 3380, 3384, 3388, 3392, 3396, 3400, 3404, 3408, 3412, 3416, 3420, 3424, 3428, 3432, 3436, 3440, 3444, 3448, 3452, 3456, 3460, 3464, 3468, 3472, 3476, 3480, 3484, 3488, 3492, 3496, 3500, 3504, 3508, 3512, 3516, 3520, 3524, 3528, 3532, 3536, 3540, 3544, 3548, 3552, 3556, 3560, 3564, 3568, 3572, 3576, 3580, 3584, 3588, 3592, 3596, 3600, 3604, 3608, 3612, 3616, 3620, 3624, 3628, 3632, 3636, 3640, 3644, 3648, 3652, 3656, 3660, 3664, 3668, 3672, 3676, 3680, 3684, 3688, 3692, 3696, 3700, 3704, 3708, 3712, 3716, 3720, 3724, 3728, 3732, 3736, 3740, 3744, 3748, 3752, 3756, 3760, 3764, 3768, 3772, 3776, 3780, 3784, 3788, 3792, 3796, 3800, 3804, 3808, 3812, 3816, 3820, 3824, 3828, 3832, 3836, 3840, 3844, 3848, 3852, 3856, 3860, 3864, 3868, 3872, 3876, 3880, 3884, 3888, 3892, 3896, 3900, 3904, 3908, 3912, 3916, 3920, 3924, 3928, 3932, 3936, 3940, 3944, 3948, 3952, 3956, 3960, 3964, 3968, 3972, 3976, 3980, 3984, 3988, 3992, 3996, 4000, 4004, 4008, 4012, 4016, 4020, 4024, 4028, 4032, 4036, 4040, 4044, 4048, 4052, 4056, 4060, 4064, 4068, 4072, 4076, 4080, 4084, 4088, 4092, 4096, 4100, 4104, 4108, 4112, 4116, 4120, 4124, 4128, 4132, 4136, 4140, 4144, 4148, 4152, 4156, 4160, 4164, 4168, 4172, 4176, 4180, 4184, 4188, 4192, 4196, 4200, 4204, 4208, 4212, 4216, 4220, 4224, 4228, 4232, 4236, 4240, 4244, 4248, 4252, 4256, 4260, 4264, 4268, 4272, 4276, 4280, 4284, 4288, 4292, 4296, 4300, 4304, 4308, 4312, 4316, 4320, 4324, 4328, 4332, 4336, 4340, 4344, 4348, 4352, 4356, 4360, 4364, 4368, 4372, 4376, 4380, 4384, 4388, 4392, 4396, 4400, 4404, 4408, 4412, 4416, 4420, 4424, 4428, 4432, 4436, 4440, 4444, 4448, 4452, 4456, 4460, 4464, 4468, 4472, 4476, 4480, 4484, 4488, 4492, 4496, 4500, 4504, 4508, 4512, 4516, 4520, 4524, 4528, 4532, 4536, 4540, 4544, 4548, 4552, 4556, 4560, 4564, 4568, 4572, 4576, 4580, 4584, 4588, 4592, 4596, 4600, 4604, 4608, 4612, 4616, 4620, 4624, 4628, 4632, 4636, 4640, 4644, 4648, 4652, 4656, 4660, 4664, 4668, 4672, 4676, 4680, 4684, 4688, 4692, 4696,